CYTO-EMBRYOLOGICAL STUDIES OF SEA URCHINS. III. ROLE OF THE SECONDARY MESENCHYME CELLS IN THE FORMATION OF THE PRIMITIVE GUT IN SEA URCHIN LARVAE

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Analyses of the mechanism of gastrulation in the past have been based either on observations of the normal process or on studies of the effect of LiCl. When one proceeds along the first line, it is rather difficult to reach a decisive conclusion. When one selects the second approach, in addition to the failure of gastrulation, another, more profound, factor of changes in differentiating potency is introduced. Yet, since lithium treatment and exogastrulation are so consistently associated, there is a tendency to equate lithium treatment with the analysis of the mechanism of gastrulation. Obviously handling two variables simultaneously is a rather dangerous procedure. In a strict sense, therefore, before attacking the lithium effect, exogastrulation not accompanied by a change in differentiation capacity should be studied. This attitude is observed in the present paper.

Normal Process of Gastrulation

Concerning the mode of gastrulation in sea urchins, the views of such earlier workers as Herbst (1893, 1896), Morgan (1896), Schmidt (1904) can roughly be summarized as saying that the endodermal plate of the beginning gastrula gives rise only to the tip of the archenteron, while the blastular wall encircling the original endodermal plate invaginates, completing the formation of the gut. These investigators believed that the volume of the larva actually decreases during gastrulation because of the inward migration of so much of the blastular wall.

On the other hand, Hörstadius (1935, 1936, 1939) showed by a vital staining technique that the presumptive archenteron takes its origin from the veg_2-disk of the 64-cell stage. At the beginning of gastrulation, this entire mass invaginates and the gut is formed by the elongation of the mass. Hörstadius further asserted, in contrast to the conclusion of the previous workers, that the volume of the larva increases during gastrulation.

Although the process of gastrulation can be observed fairly clearly in the living larvae of any transparent forms, the observation can be further supplemented by following the development of individual larvae reared in a simple moist chamber as described below.

From a piece of tissue paper, the thickness of which is a little less than that of the larva, a square piece slightly smaller than a cover slip is cut out and a hole

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several millimeters in diameter is made at the center. After wetting the paper with sea water on a slide, a droplet of sea water containing one larva is placed at the center of the hole (but not touching the paper) and a cover glass is slowly laid down. The wet paper prevents evaporation and the droplet is the culture medium for the larva. If the entire preparation is put into a still larger moist chamber, or if distilled water is occasionally added to the paper (these methods are preferable to sealing the cover slip with vaseline), it is possible to rear the larva from the gastrula to the pluteus stage. In this small space, as the larva swims rather slowly,

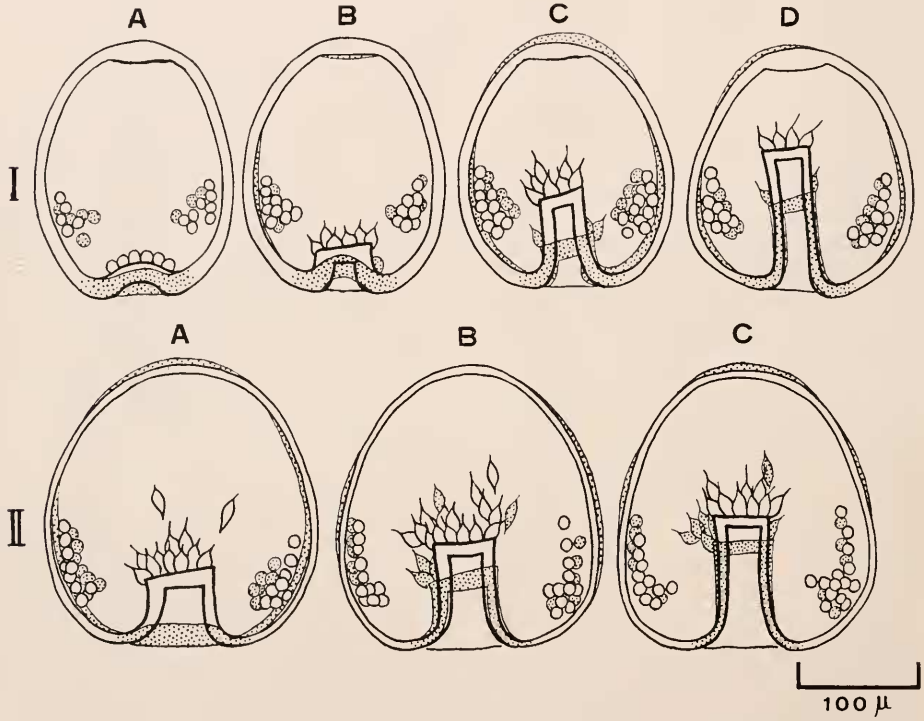

FIGURE 1. Superimposed contour-tracings of photographs of a single larva through the gastrulation process. Dotted drawings represent preceding stages and open drawings are succeeding stages. The upper row: *Clypeaster japonicus;* the lower row: *Mespilia globulus.*

photographs of various views of the larva can be made with a shutter speed of $\frac{1}{50}$ second. Further, in such droplets, although development is slowed down to some extent, no sign of morphological abnormalities was detected.

Figure 1 shows superimposed tracings of a series of photographs, taken at appropriate intervals, of the larvae of two kinds of sea urchin. As can be seen from the figure, throughout the period from the stage of endodermal plate formation to the completion of gastrulation, the contours of the larvae and the positions of the primary mesenchyme cells hardly change, while the archenterons increase their lengths considerably. Moreover, it will be noticed that as the archenteron elongates, the thickness of its wall decreases. The authors believe that this fact sup-

ports the conclusion of Hörstadius mentioned. The only discrepancy found is that the volume of the larva remains constant during the gastrulation process, and it is only after the completion of gastrulation that the volume increase takes place.

From both Hörstadius' results and these observations of the authors, the following facts seem to be certain:

1) All the material which will form the archenteron is first laid down as an endodermal plate preceding gastrulation.

2) At the beginning of gastrulation, the entire mass of this endodermal plate invaginates *in toto*.

3) The formation of the primitive gut is due to the stretching of the original endodermal plate, with no other elements included.

Formation of Pseudopodia by the Secondary Mesenchyme Cells

Soon after the onset of invagination of the endodermal plate, the secondary mesenchyme cells, differentiating at the tip of the archenteron, begin to send out fine pseudopodia.[3] These pseudopodia elongate toward the wall of the animal side of the larva, and by the time the archenteron attains a length ½–⅔ the diameter of the blastocoel, the ends of the pseudopodia are attached to the blastular wall either directly or by the mediation of a few cells arranged on top of the others.

After the establishment of the connection between the blastular wall and the pseudopodia, larvae are occasionally found in which the part of the blastular wall where the pseudopodia are attached is pulled in. In *Pseudocentrotus* larvae this effect is very conspicuous. In mid- or late gastrula stage, they frequently acquire two indentations, one on the animal side where the pseudopodia are anchored, and the other, the blastopore, on the vegetal side; thus the entire larvae somewhat simulate the shape of a mammalian red blood cell (see also Okazaki).

Moreover, if the shape of the tip of the archenteron is carefully observed, it will be seen that it seldom has a rounded contour, but it is of an edgy or squarish shape resembling a suspended fabric. In case a group of pseudopodia develops better on one side, the roof of the archenteron is lifted up higher toward the animal pole on that side. All these facts give the impression that the archenteric roof is being pulled toward the animal pole by the pseudopodia of the secondary mesenchyme cells. This interpretation is further consolidated by the following several experiments.

Injection of Oil Droplets into the Blastocoel

In order to find out whether mesenchymal pseudopodia are actually capable of pulling an object, the simplest way will be to introduce an easily movable object into the blastocoel. For this purpose, oil droplets were micro-injected into the blastocoels of newly hatched blastulae. When the primary mesenchyme cells appear, regardless of the position in the blastocoel of the oil droplets, they are caught by the primary mesenchyme cells. However, when the secondary mesenchyme cells are formed, their traction force is apparently stronger than that of the primary

[3] The time of appearance of the pseudopodia differs from one species to another. In *Mespilia*, in which they appear earliest, the pseudopodia are formed immediately after the initiation of invagination; in *Pseudocentrotus*, in which they appear late, the pseudopodia can be recognized clearly only after the archenteron reaches ⅓ of the distance across the blastocoel.

mesenchyme cells so that by the time the mid- or late gastrula stage is reached, the oil droplets are relayed over to the secondary mesenchyme cells and are drawn toward the archenteric tip. Some photographs are shown in Figure 2. It should be pointed out that since the injected oil drop is immediately surrounded by a protein film, the pseudopodia have no difficulty in getting hold of it.

Now having seen that the basic conditions are fulfilled, let us proceed to examine whether or not the pseudopodia are actually pulling up the archenteron.

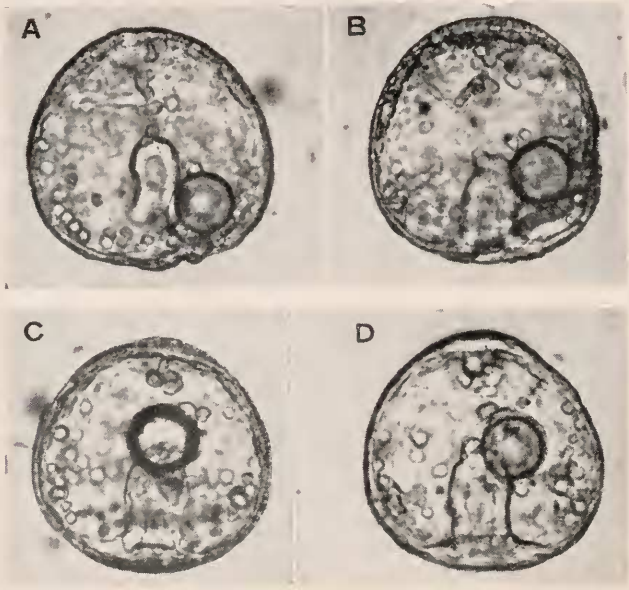

FIGURE 2. Microphotographs of *Clypeaster* larvae with oil drops injected into the blastocoels. A. Early stage. Oil droplet is caught by the pseudopodia of the primary mesenchyme cells. B. Intermediate stage. The oil is picked up by the pseudopodia of the secondary mesenchyme cells also. C, D. Later stages. As the pull by the secondary mesenchyme cells is stronger, the oil is removed from the primary mesenchyme cells and is drawn toward the archenteric tip.

EFFECTS OF EXPERIMENTAL INCREASE OF THE BLASTOCOELIC VOLUME

If the tip of the archenteron is actually being pulled up toward the animal pole by pseudopodia which have anchored themselves on the blastular wall, the pulling force will be increased if the blastocoelic volume is increased. This increased pull, in turn, would stretch the archenteron making it longer. Moore (1939, 1940) and Dan (1952) have reported that since the sea urchin blastular wall loses permeability to sucrose molecules after the tenth cleavage cycle (*i.e.*, at the time of hatching), if larvae cultured in a sucrose-containing medium are transferred to pure sea water after hatching, they become considerably inflated as the result of an abnormally high internal osmotic pressure developed by the sucrose molecules entrapped in the blastocoel.

Repetition of the sugar procedure on *Clypeaster japonicus* and *Mespilia globulus* gives not only a confirmatory result concerning the above expectation but it further

gives rise to exogastrulae in the proportion given in Table I. Figure 3 illustrates superimposed tracings of photographs of individual larvae of *Mespilia* during the process of inflation and of exogastrulation. As can be seen in the figure, during the few minutes following the transference from sucrose sea water to pure sea water when the larva is increasing in size, the pseudopodia are stretched and at the same time, the archenteron actually lengthens (Fig. 3, B, C). However, when the volume is further increased, the pseudopodia or chains of mesenchyme cells extending between the gastrular wall and the tip of the archenteron are broken and the cells round up. Under this circumstance, the archenteron begins to be pushed out of the blastocoel from its basal part (Fig. 3, F and Fig. 4).

Whenever the archenteric tip is wide and flat-topped, it was invariably found that some of the pseudopodia are anchored to the lateral wall instead of to the animal pole region so that they are pulling the archenteric tip sideways (Fig. 3, D).

TABLE I

Percentages of normal, entoexo- and exogastrulae obtained by inflation of the blastocoel by sugar treatment in Clypeaster japonicus and Mespilia globulus

Species	Stage of returning from sugar solution to sea water	10% sugar s.w.→ Natural s.w.			20% sugar s.w.→ Natural s.w.			30% sugar s.w.→ Natural s.w.		
		Invaginated form	Ento-exo-gastrula	Exo-gastrula	Invaginated form	Ento-exo-gastrula	Exo-gastrula	Invaginated form	Ento-exo-gastrula	Exo-gastrula
Clypeaster	Early gastrula	—	—	—	72.5%	15%	12.5%	92%	5.5%	2.5%
	Mid gastrula	—	—	—	65	14.5	20.5	25	21	54
Mespilia	Early gastrula	67%	5%	28%	43	12	45	—	—	—
	Mid gastrula	68	10	22	30	12	58	—	—	—
	Early gastrula	74	8	18	65	8	27	—	—	—
	Mid gastrula	28	32	40	18	24	58	—	—	—
	Late gastrula	69	26	5	62	25	13	—	—	—

At any rate, as long as the pseudopodial connection is intact, the archenteric tip takes an edgy contour like a suspended fabric, while if the connection is severed, the contour rounds off (Fig. 3, E, F).

Generally speaking, secondary mesenchyme cells are amoeboid. Therefore, they are continuously extending and retracting pseudopodia, forming new ones and abolishing old ones, and some cells move along other cells. When such pseudopodia are broken as the result of blastocoelic expansion, although the secondary mesenchyme cells round up for a while, sooner or later they begin to send out new pseudopodia. In a larva in which only the basal part of the archenteron is evaginating, if a new pseudopodial connection is established in the normal position, the archenteric tip begins to be pulled back toward the animal pole once more. As a matter of fact, even in larvae in which the entire archenteron has been everted, the archenteric tip can still resume the inward movement when a connection is successfully established. Because of these secondary connections, various degrees of entoexogastrulae result in an experimental batch. Some of these are depicted in Figure 4, A–F.

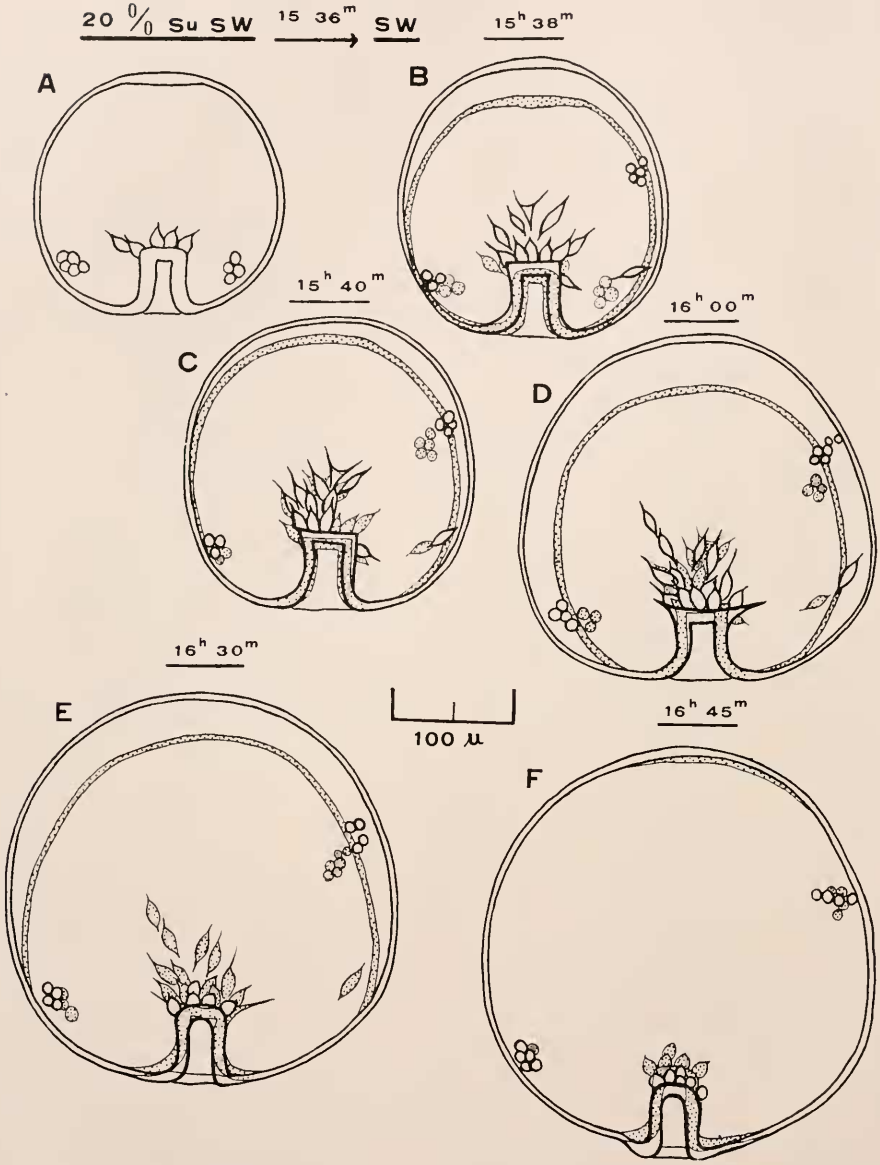

FIGURE 3. Effects of inflation of the blastocoel on the pseudopodia of the secondary mesenchyme cells and on the archenteron of *Mespilia globulus*. A. A larva in a sugar-containing sea water before transfer. B, C. Transfer to pure sea water. As a result of blastocoelic expansion, the pseudopodia are stretched, in turn pulling the archenteron longer. D. Flat-topped archenteron indicates that the pseudopodial connection to the animal pole has been severed while the connection to the side wall is still preserved. E. Rounded tip of the archenteron indicates the severance of the pseudopodial connection and the archenteron is receding. F. The secondary mesenchyme cells have also rounded up and the gut is evaginating.

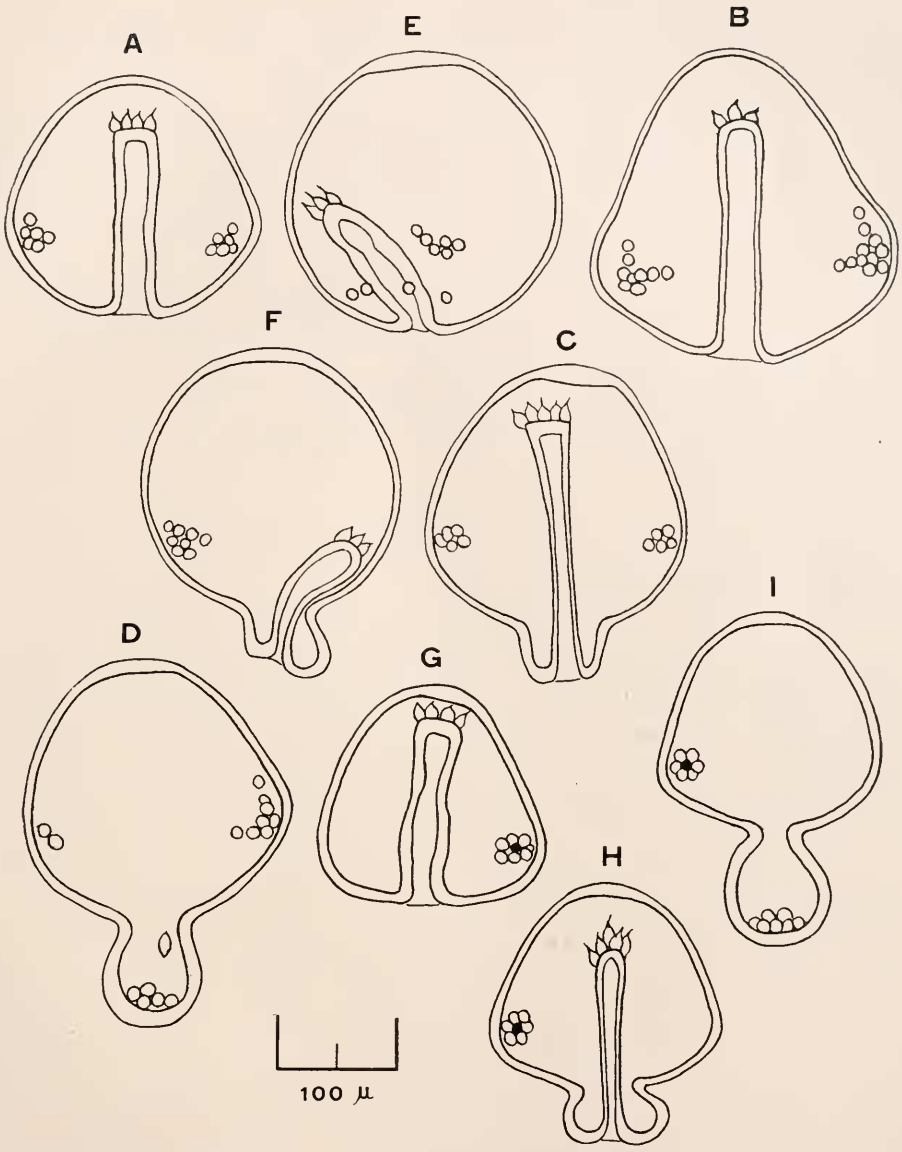

FIGURE 4. Various types of abnormality in *Clypeaster japonicus*. A-F: Transferred from sugar to pure sea water at early gastrula stage. A. Control in sugar-sea water. B. Inflated larva which has successfully invaginated. Note the height of the archenteron and the thinness of its wall. C. An entoexogastrula. The wall of the gut is thinned out considerably. D. An inflated larva with a completely evaginated gut. The wall of the gut is very thick. E. An inflated larva in which the pseudopodia have attached themselves to the side wall. F. The same as E with the gut going astray. G-I: Exposed to Ca-low condition ($\frac{1}{10}$ that of sea water) from the gastrula stage onward. G. Control for Ca-low series. H. An entoexogastrula caused by the Ca-low treatment. I. Exogastrula in $\frac{1}{10}$ Ca.

From Figure 4 it becomes evident that the completely everted archenteron of an exogastrula (D) is shorter and its wall is thicker than the control (A), while the invaginated part of the gut of an entoexogastrula is highly stretched and its wall is extremely thin (C). This result is to be expected in the latter type, since a part of the gut having been everted, the remaining fraction has to supply all the material to be pulled out to reach the animal pole. Comparison of Figures 4 A and 4 B shows that even among successfully invaginated larvae, a proportionality is evident between the size of the blastocoel and the length of the gut or the thinness of the archenteric wall.

From the above observations, the following points can be inferred. When the blastocoel is abruptly inflated by a transfer from a sucrose medium to sea water, the pseudopodia of the mesenchyme cells tend to break and the primitive gut retreats. But judging from cases of entoexogastrulae, if the pseudopodial connection is re-established, its mild but continued pull can eventually pull out the primitive gut so that it becomes longer than normal (C). The short, bag-like shape of the completely everted archenteron of an exogastrula (D) must be produced under the

TABLE II

Percentages of normal, entoexo- and exogastrulae caused by Ca-low treatment in Clypeaster

S.W.....1/10 Ca

Stage of transfer	Experiment 1			Experiment 2		
	Invaginated	Entoexogastrula	Exogastrula	Invaginated	Entoexogastrula	Exogastrula
Early gastrula	31%	16%	53%	18%	0%	82%
Mid gastrula	39	34	27	74	7	19
Late gastrula	87	10	3	96	2	2
Post gastrula	97	3	0	100	0	0

complete absence of such an extrinsic traction. By the time the normal larva reaches the pyramid stage, however, the everted gut of the exogastrula grows in length to a limited extent and eventually exhibits a perfect tripartite formation. This fact indicates that even in the everted gut, tissue differentiation can take place perfectly, although such a gut never assumes the cylindrical shape typical of normal gastrulae of an advanced stage. This, in turn, indicates that the typical cylindrical shape and squarish tip of the normal archenteron is brought about by the pulling force of the pseudopodia. On the other hand, an abnormally stretched archenteron of an entoexogastrula often breaks in the thinnest part, and the upper portion goes to the animal pole while the basal portion evaginates completely. The result is a larva which has a stomodaeum and a pharynx at the normal position but with the stomach and intestine everted. This is obviously a case in which the primitive gut has yielded to the pulling force.

Among larvae which have been subjected to the sucrose treatment, such forms as those shown in Figure 4 E and 4 F are also encountered, in which the tip of the archenteron has failed to reach the stomodaeum although the gut as a whole remained inside the blastocoel. The cause for this lies in the fact that when new pseudopodia were formed, they attached themselves to the side wall instead of to

the wall of the animal pole and as a consequence, the archenteric tip is led to a wrong destination.

Suppression of Pseudopodial Formation of the Mesenchyme Cells

From what has been said, it can be expected that exogastrulae will also result if the pseudopodia are made to retract instead of being broken. In order to cause already formed pseudopodia to disappear, there are three methods available.

1) In *Clypeaster,* if the larvae are transferred to Ca-low sea water ($\frac{1}{10}$ Ca concentration of sea water) at the gastrula stage when pseudopodia are being formed by the secondary mesenchyme cells, the pseudopodia disappear in some of the larvae and exo- and entoexogastrulae similar to those obtained by the inflation technique are produced (Fig. 4, G–I). The frequency of their occurrence is given in Table II. Closer tracing of the development of a single larva reveals that the suppressing effect of the Ca-low medium works rather slowly and for a few hours pseudopodia can be seen, during which time the archenteron keeps on elongating little by little. But as soon as the pseudopodia disappear completely, the archenteron begins to retreat. The correlation between the two is quite definite, as will be seen in Figure 5, I.

In the Ca-low batch, various gradations of suppression are seen: complete suppression giving exogastrulae (Fig. 5, I), resumption of pseudopodia formation resulting in entoexogastrulae (II), formation of very slender and weak pseudopodia enough to prevent exogastrulation but failing to bring the archenteric tip to the stomodaeum (III), and finally quasi-normal larvae (Fig. 5, IV). In the III class of larvae, the secondary mesenchyme cells form an unpaired coelom at the tip of the primitive gut, thus preventing the union between the archenteron and the stomodaeum.

2) In *Mespilia,* if the gastrulae are exposed to pancreatin (0.5 to 0.125% in sea water), the pseudopodia are abolished in a short time and exogastrulae are formed (Fig. 6). The percentages of entoexo- and exogastrulae are given in Table III. Besides the suppression of the pseudopodia, the pancreatin has secondary effects such as decreasing the larval volume and causing the blastular wall to wrinkle. This latter effect is presumably due to the action of pancreatin on the intercellular matrix and on the hyaline layer. However, concerning the hyaline layer, Moore (1952) has shown that this layer does not play an essential role in the gastrulation process. Because of these secondary effects, it is impossible to rear the larvae in the pancreatin solution indefinitely. However, if they are returned to sea water in time, larvae similar to those shown in Figures 4 and 5 for *Clypeaster* can develop.

3) Among *Mespilia* larvae kept in normal sea water, it sometimes happens that gastrulation proceeds seemingly normally during the early phase, but later spontaneously deviates from the normal course and leads to exo- or entoexogastrula formation. Even in such spontaneous cases, coincidence in the time of pseudopodial disappearance and evagination is always recognized.

Total Inhibition of Pseudopodia Formation

This can be achieved in *Pseudocentrotus* larvae by rearing them in a Ca-low medium ($\frac{1}{20}$ to $\frac{1}{10}$ normal Ca level) from an early stage. In this case, no sign

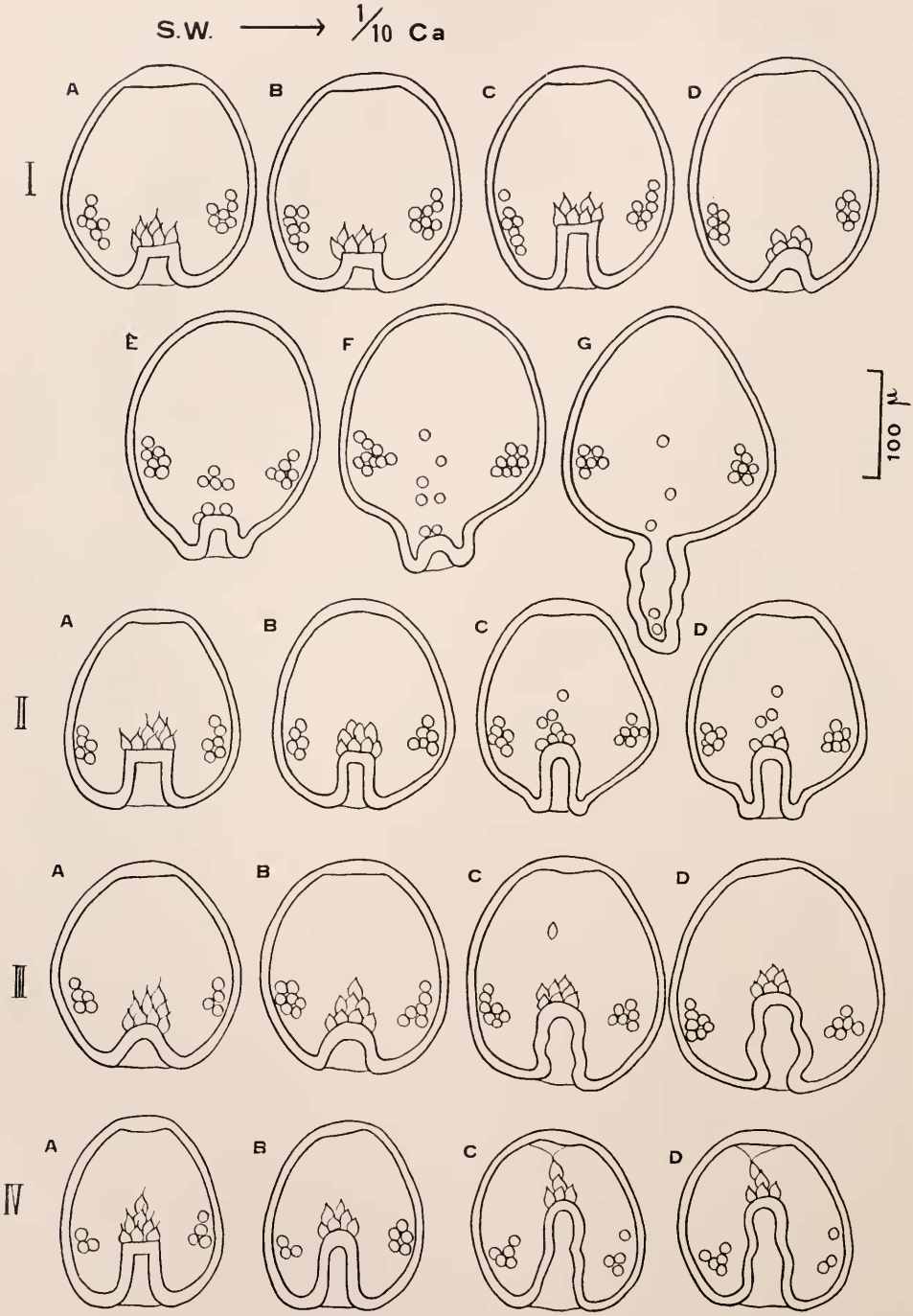

FIGURE 5.

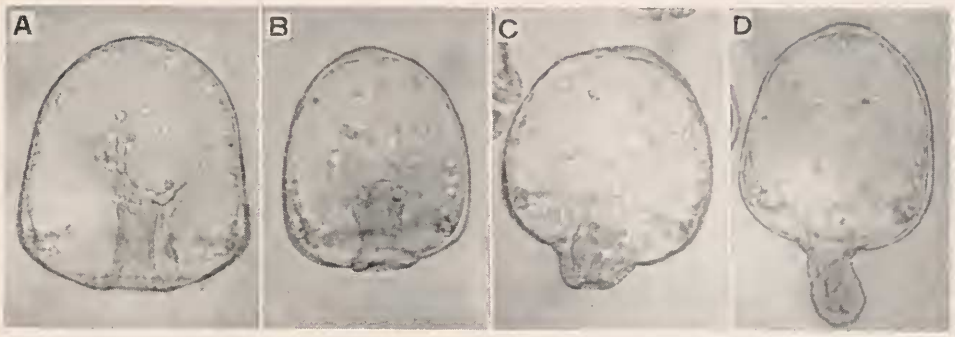

FIGURE 6. Course of exogastrula formation by pancreatin-sea water in *Mespilia globulus*.

of pseudopodia can be found and the archenteron extends ⅓ to ½ of the way across the blastocoel and stops there. By the time normal larvae in sea water have completed gastrulation, the archenterons of the larvae in the Ca-low medium gradually begin to retreat and they finally form exogastrulae. (A more detailed account of this process will be given in another paper by Okazaki.)

TABLE III

Percentages of normal, entoexo- and exogastrulae caused by pancreatin treatment in Mespilia

Stage of transfer	Pancreatin concentration								
	0.5%			0.25%			0.125%		
	Invaginated	Entoexogastrula	Exogastrula	Invaginated	Entoexogastrula	Exogastrula	Invaginated	Entoexogastrula	Exogastrula
Early gastrula	3%	15%	82%	12%	10%	78%	37%	7%	56%
Mid gastrula	12	18	70	24	26	50	34	6	60
Late gastrula	40	14	46	13	19	68	55	10	35
Post gastrula	88	7	5	91	5	4	89	2	9

DISCUSSION

The experimental results reported in this paper have shown that the pseudopodia of the secondary mesenchyme cells play an indispensable role in the formation of the primitive gut of sea urchin larvae. However, it is admitted that the pulling of the mesenchymal pseudopodia alone is not sufficient to account for all the power required to make a young endodermal plate change into the cylindrical gut of a late gastrula. For one thing, the endodermal plate begins to invaginate at the very be-

FIGURE 5. Suppression of the pseudopodia of the secondary mesenchyme cells of *Clypeaster japonicus* by 1/10 Ca that of sea water. The first figure (A) for each series is a larva immediately before transfer to the Ca-low medium. I. Complete suppression and resulting exogastrula formation. II. Partial recovery of the pseudopodia leading to an entoexogastrula. III. Feeble pseudopodia just strong enough to keep the archenteron from eversion but failing to draw it to the stomodaeal position. Such a larva acquires an unpaired coelom intervening between the stomodaeum and the archenteron. IV. A seemingly perfect larva in 1/10 Ca.

ginning of gastrulation, before the secondary mesenchyme cells start to form pseudopodia. Consequently, the pseudopodia cannot in any way determine whether the endodermal plate invaginates or evaginates. For another thing, such an endodermal plate can elongate by its own power without the cooperation of the pseudopodia. Probably it is justifiable to think that the maximal limit of this autonomous elongation is shown by the length of completely everted gut of an exogastrula.

Then what is the factor which causes this autonomous elongation of the endodermal plate? As has been realized by many previous workers, the only clue available at present is the change in shape of the cells composing the endodermal plate. While they are in the endodermal plate, they are tall and cylindrical. But as the invagination proceeds, they flatten out laterally. This change takes place even when the endodermal plate evaginates from the beginning. However, a point of importance is the fact that this flattening normally occurs in such a direction that it can be magnified by the pull by the pseudopodia.

Thus, the authors view the situation as follows: within the process of gut formation, three factors must be operating; (1) a factor responsible for the initiation of the invagination of the endodermal plate, (2) a process bringing about the form change of the constituent cells of the endodermal plate and (3) the traction exerted by the pseudopodia. During the early gastrula stage, the first two are acting while in the late gastrula stage, the second and third must be cooperating.

Concerning the first factor, which initiates invagination in the endodermal plate, various views have been expressed. Long ago Rhumbler (1902) attempted an analysis of the process on the premise that three factors—(a) pressure within the blastular wall resulting from increase in cell number, (b) pressure by a confining membrane and (c) absorption of blastocoelic fluid—might be playing some role. But after showing that none of these was involved, he tried to attribute the cause of inpocketing to surface tension changes in the vegetal cells. Morgan (1927) and a few others considered that it might be due to the pressure of dividing cells acting in a plane passing through the apical and basal poles of the embryo. Agrell's recently published opinion (1954) hints at a similar possibility. However, Morgan's idea was negated by Moore (1939). Moore himself contends that the invaginating force resides within the endodermal plate itself and this force decreases along the radial gradient with a maximum at the center. He attempted to calculate this force, using the results of his sugar experiments (1941).

Since then, Lewis (1947) has suggested for amphibian invagination that the superficial gel layer might be playing a role. Moore (1952) went on to test the validity of Lewis' idea in sea urchins and concluded that the hyaline layer of sea urchins is not essential for gastrulation (see also Dan, 1952). Gustafson and Lenique (1952) try to explain it by an increased pressure within the wall caused by a structural protein which is derived from mitochondria.

With respect to the form change of the cells making up the endodermal plate, Gustafson suggests the same explanation, that is, stretch by a structural protein. However, it must not be forgotten that besides simply elongating, such a gut is also able to self-differentiate.

Finally, the process of development of a functional digestive tract in sea urchin larvae requires a fusion between the tip of the archenteron and the gastrular wall at the stomodeal position. The authors believe that the observations presented in

this paper show conclusively that this is accomplished by the directing and pulling activity of the secondary mesenchyme cells, before they begin their main task of forming the coelom.

However, the authors do not hesitate to admit that the elucidation of the mechanism of the gastrulation process is still remote from a solution. What causes the formation of the endodermal plate to begin with? What factor initiates its invagination? What factors are involved in the autonomous elongation and self-differentiation of the primitive gut? How do the secondary mesenchyme cells know the site of the stomodaeum? All these important questions are left unanswered.

Summary

1. The first half of the gastrulation process in sea urchins begins with an *in toto* invagination of the endodermal plate, followed by its stretching.

2. In the second half of the process, pseudopodia are sent out by the secondary mesenchyme cells toward the animal pole. These pseudopodia attach to the blastular wall and pull the archenteron up toward that pole.

3. The pulling capacity of the pseudopodia is shown by the displacement toward the secondary mesenchyme cells of oil droplets injected in the blastocoel.

4. When the pseudopodial connection is artificially severed by one of the following methods, exogastrulae result:

a) Blastocoelic expansion after sucrose treatment (*Mespilia globulus, Clypeaster japonicus*).

b) Ca-low treatment (*Clypeaster, Pseudocentrotus depressus*).

c) Pancreatin treatment (*Mespilia*).

5. Abnormal invagination in various degrees brought about by the above treatments can be accounted for by differences in their effects on the pseudopodia formation.

6. Pseudopodia of the secondary mesenchyme cells are indispensable in the latter half of the gastrulation process in order to produce normal embryos.

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